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Annual Progress Report

Report prepared by: Manfred M. Mayer

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Contractor: Johns Hopkins University

Principal Investigator: Manfred M. Mayer

Assistants: L. Levine, Sc.D., Instructor

J. H. Rapp, Sc.M., Student

A. Alvin Marucci, Sc.M., Student

F. A. H. Rice, Ph.D., Asst. Professor

Title of Project: Nature of Cytotoxic Reactions Mediated by Antibody
and Complement, and Related Phenomena

Objectives: Investigation of fundamental mechanisms of antibody and
complement action.

Abstract of Results:

a. Since start of project:

Most of the work conducted so far on the nature of cytotoxic reactions mediated by antibody and complement has been concerned with the immune lysis of sheep erythrocytes, since this reaction serves as a convenient model from the standpoint of availability of reagents as well as the feasibility of precise measurement. However, in accord with the broad objectives of our program some studies have been conducted with Treponema pallidum as well as with typhoid bacilli.

Summary of Results on Hemolysis by Antibody and Complement:

1) From kinetic observations it has been deduced that hemolytic antibody acts like an enzyme in the sense that it exhibits turnover. This has been confirmed by direct demonstration of the reversibility of the red cell-antibody combination and the resultant transfer of antibody from cell to cell during the hemolytic process.

2) A kinetic method for the titration of the hemolytic activity of antibody has been developed.

3) A precise quantitative hemolysis-inhibition procedure has been devised.

4) In pursuit of the hypothesis that the erythrocyte antigen which combines with hemolytic antibody may represent the substrate on which antibody and complement act, we have isolated an alcohol-soluble, heat-stable hapten from the sheep erythrocyte membrane (designated F-substance) and have succeeded in obtaining this material in partially purified form. F-substance has the chemical properties of a lipo-polysaccharide

With the aid of this hapten it has been possible to make precise quantitative measurements of the amount of antibody, in weight units, which is required to mediate the action of complement on the red cell surface.

5) By ultracentrifugal measurements it has been determined that the hemolytic antibody reactive with this hapten possesses a molecular weight of approximately 1 million, and from this, as well as from the antibody weight determinations, it could be calculated that for optimal reactivity with complement about 1000 molecules of antibody per erythrocyte are required. However, as few as 6 molecules of hemolytic antibody are sufficient for mediating the lysis of a single red cell in conjunction with complement.

6) This observation is in conflict with the Alberty-Baldwin theory of immune hemolysis according to which at least 10 molecules of antibody are required for the lysis of a single red cell. We have been able to develop two other, independent lines of evidence which also indicate that the Alberty-Baldwin theory is erroneous: First, it has been observed that in a system containing constant complement and varying numbers of erythrocytes, the number of red cells hemolyzed is constant (i.e. independent of the total number of cells in the system), provided the degree of lysis is below 50 per cent. This suggests that hemolysis by antibody and complement is a single-hit reaction. The second line of evidence has emerged from kinetic studies of the nature of the lag or induction period. In line with the Alberty-Baldwin theory, which postulates that immune hemolysis is a multiple-hit phenomenon, the lag period was explained in terms of the time required for

the accumulation of at least 10 damaged sites per red cell. On the other hand, if only a single damaged site is required, an alternative explanation for the lag period must be found. Recent studies have shown that the action of complement takes place in at least 3 successive steps, and that the lag or induction period can be accounted for in this manner.

7) It has been shown that Ca^{++} is specifically required for the fixation of complement by antigen-antibody complexes, or more specifically, for the fixation of certain components of complement which we have tentatively designated C'_x . This reaction is followed by a process involving Mg^{++} and other components of complement, tentatively designated C'_y . A third reaction step has been identified which involves the transformation of the cell, previously damaged by the Ca^{++} and the Mg^{++} steps, into a ghost with release of hemoglobin. This terminal reaction requires no reactant from the fluid phase, and is considered to be an intrinsic transformation process. It takes place in approximately two minutes per cell. The temperature coefficient of this reaction is about 1.5 per 10° rise.

8) Methods have been developed for measuring the rates of the Mg^{++} and the terminal transformation reaction steps.

9) Procedures have been developed for the separation of C'_x from C'_y , and this opens the way for studying the Ca^{++} and Mg^{++} reaction steps as separate processes.

10) In an attempt to learn whether complement in conjunction with antibody exerts any effect on soluble protein antigens a study was made

of the inhibition of the enzyme urease by rabbit antibody. It was found that the degree of inhibition is a function of the molecular ratio of antibody to enzyme. No effect of complement could be demonstrated.

11) It has been found that certain high molecular weight polymers like carbowax 4000 or carbowax 6000, or polyvinyl pyrrolidone, or dextran, can be substituted for hemolytic antibody in the sense that they will mediate the lysis of erythrocytes by complement. The properties of this reaction have been studied and one of the most interesting findings is the observation that it requires approximately 5 times the concentration of Ca^{++} than the hemolytic reaction mediated by specific rabbit antibody.

12) A study has been made of the complement-fixing ability of specific antisera in relation to their content of antibody as measured in weight units. It has been found that the specific complement-fixing ability, i.e. amount of complement fixed per milligram of antibody, may vary by a factor of 4 depending on the extent of immunization of the animal. This is of considerable interest in connection with studies of the properties of antibody, and it has led us to experiments aimed at the characterization of antibody-antigen combination in thermodynamic terms.

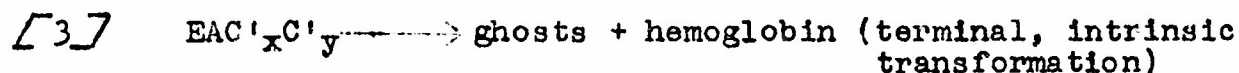
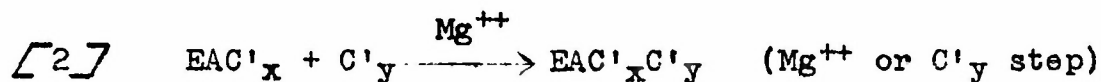
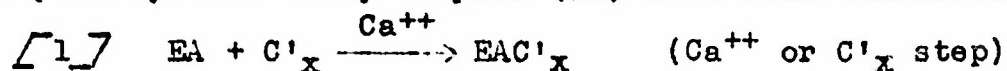
13) Progress has been made in elucidating the kinetics of antibody-complement action by demonstrating that in a system containing excess antibody and limited complement the rate of reaction is controlled by the Mg^{++} step, while in a system containing excess complement and

limited antibody the pace-making reaction is the Ca^{++} step, i.e. fixation of C'_x .

14) A quantitative specific test for horse meat in cooked meat products has been developed.

b. Abstract of results during current report period:

The following three reaction steps in the action of complement on the erythrocyte-antibody complex (EA) have been demonstrated:



C'_x and C'_y represent certain components of complement.

Methods have been devised for determining the rates of reactions $\boxed{2}$ and $\boxed{3}$ by the use of sampling techniques which block these reaction steps. Reaction $\boxed{2}$ is blocked by dilution and chilling, while reaction $\boxed{3}$ is arrested by separation of the cells from the fluid phase in the centrifuge. The details of these methods are described in the fourth paper of the series on "Kinetic Studies on Immune Hemolysis".

Reaction $\boxed{1}$ has a much lower temperature coefficient than reaction $\boxed{2}$. Therefore, it is possible to go through reaction $\boxed{1}$ at a low temperature (i.e. 0-10°C) without appreciable progress of reaction $\boxed{2}$. By the use of controlled temperature, as well as a reaction system containing Ca^{++} , but lacking Mg^{++} , it is possible to go

through step [1], while step [2] is blocked. This results in formation of the complex EAC'_x which can be isolated by centrifugal separation of the cells and removal of the fluid phase. C'_x is firmly fixed to the complex EA and therefore the cells in the state EAC'_x may be washed for complete removal of C'_y with the fluid phase. Similarly, it has been found possible to remove C'_x from whole complement by treatment with specific precipitates in the presence of Ca^{++} , but absence of Mg^{++} , and in the cold. This yields a reagent containing C'_y , but lacking C'_x . Upon admixture of C'_y with EAC'_x , reaction [2] ensues, provided Mg^{++} is supplied.

Preliminary studies suggest that EAC'_x prepared at a low temperature actually consists of a mixture of cells in the sense that some cells are in the state $EAC'_{1,4,2}$, while other cells are in the state $EAC'_{1,4}$ (the subscripts refer to the recognized components of complement). Similarly, C'_y prepared at a low temperature contains C'_2 and C'_3 . On the other hand, if reaction [1] is carried out at $37^\circ C$ with a dilution of C' which gives no lysis with Ca^{++} alone, but would cause lysis if Mg^{++} were also present, the complex EAC'_x , which is formed, appears to consist entirely of $EAC'_{1,2,4}$. At present our efforts are directed toward a definition of the conditions under which C'_2 reacts with the complex $EAC'_{1,4}$, with the objective of studying the subsequent reaction of this complex with C'_3 as a single reaction step. From the data available at present it is the C'_3 reaction step which requires Mg^{++} .

A radio-tracer method for the quantitative determination of antibody on a microlevel:

The objectives and general mode of approach of this method have been described in the previous report (July 1953). From the few, brief publications in this field it seemed that no serious difficulty would be encountered in developing this method. However, we have come up against some rather formidable obstacles. Foremost, among these, it has been found that the specific radioactivity (counts per minute per milligram of antibody nitrogen) of the antibody in a washed specific precipitate is always significantly lower than the specific radioactivity of gamma globulin prepared either by Cohn's method 10, by 33% ammonium sulfate precipitation (Jager et.al.), or by the fractionation method of Sternberger, which involves the use of ammonium sulfate between 30% and 35% saturation. Furthermore, the same difficulty has been encountered with all fractions prepared by the following method: A globulin precipitate made at 40% saturation with ammonium sulfate was successively extracted with ammonium sulfate solutions of 38%, 36%, 34% saturation, etc. down to 24%. The specific radioactivity of all of these fractions was higher than that of the antibody precipitated from them, just as for antibody precipitated from whole serum or from the gamma globulin preparations referred to previously. It is therefore evident that all of these fractions, as well as whole serum contain material which iodinate at a higher rate than the antibody.

Moreover, the specific radioactivity of the antibody varies with

the nature of the material from which it is precipitated. Antibody precipitated from whole serum shows a much higher specific radioactivity than antibody precipitated from the various globulin fractions which have been examined. It is possible that antibody may be heterogeneous with respect to iodination rate, but, if this were the sole difficulty, it would be expected that the specific radioactivity of antibody precipitated from whole serum should have a value intermediate with respect to that of antibody precipitated from the various globulin fractions.

Another possible explanation of our results might be co-precipitation and incorporation in the specific precipitate of non-antibody material possessing a high iodination rate. Co-precipitation experiments conducted so far, however, do not indicate that this is a major source of error. Therefore, the problem is not yet resolved.

The closest we have been able to come in regard to the objective of obtaining a protein fraction from serum which exhibits a specific radioactivity close to that of antibody precipitated from whole serum, is the use of a globulin fraction precipitated at 33% saturation with ammonium sulfate. This material showed the same specific radioactivity (within 1%) as that of the antibody precipitated from the whole serum.

F-hapten-rabbit antibody system:

In continuation of earlier work, precipitin analyses of the F-hapten-rabbit antibody system have been performed at 0-2°C and at 37°C. Somewhat higher results have been obtained in the cold, but at least one week is required for complete precipitation at 0-2°C. Under these

conditions it has been found possible to obtain very good agreement between percentage of antibody precipitated (as measured on a weight basis) and percentage of hemolytic antibody neutralized (as determined by hemolysis inhibition assay). An experiment of this type with anti-serum pool Q is tabulated below:

Tube No.	1	2	3	4	5	6
F-hapten N, microgm./ml.serum	2.7	5.4	10.1	18.0	33.8	67.5
Ab.N./ml. microgms.	35	67	120	190	238	238
% Ab. ppt'd.	15	28	51	80	100	100
% Hemolytic activity neutralized	15	27	48	81	95	97

The agreement between neutralization of activity and precipitation of antibody on a weight basis permits the conclusion that all the hemolytic antibody in this type of antiserum (prepared by inoculation of rabbits with boiled sheep erythrocyte stromata) is precipitable by the F-hapten, and that the F-hapten is homogeneous, at least in respect to immunological specificity; i.e. the F-hapten-rabbit antibody system used here behaves as a single antigen-antibody reaction. The possibility that the antiserum contains non-hemolytic antibody which is non-reactive with the F-hapten cannot be ruled out from these data, but this point will be checked in the future by means of the radio-iodinated antibody technique described above.

Plans for Future

Immediate:

With the recognition of the Ca^{++} and Mg^{++} reaction steps in the action of complement on sensitized erythrocytes it becomes necessary to study the role of the recognized four components of complement in these two processes. As indicated above, experiments along these lines have been initiated. It is already quite clear that components 1 and/or 4 are involved in the Ca^{++} step, while component 3 functions in the Mg^{++} step. However, the role of the 2nd component has not been clarified adequately as yet. We are attempting at the present time to learn under what conditions the action of the 2nd component takes place, so that experiments can be designed in which sensitized cells can be taken through the reaction steps involving the 1st, 4th and 2nd component of complement so as to enable us to study the subsequent reaction step involving the 3rd component. This is considered a necessary preliminary to any attempt at the isolation of the 3rd component and study of its properties.

Conventional titrations of the components of complement are seriously lacking in specificity as well as precision, and we are attempting to develop entirely new techniques to overcome these difficulties. It seems to us that there is every reason to attack the problem backwards, i.e. to focus attention on the 3rd component in the immediate future and then proceed stepwise to the mode of action of the 2nd component, 4th component and finally the 1st component. The eventual objective is, of course, the isolation of each of the components, the study of its properties and the elucidation of its mode

of action. From the information available at present it is quite clear that we are dealing with a series of successive reaction processes, and that therefore the problem has to be attacked in a stepwise fashion.

The work on radio-iodinated antibody will be continued in an effort to refine the method to the point where it can be used for the measurement of antibody weight combining with red cells, bacteria, viruses, etc. Of immediate concern to us, of course, is the application of this method to the red cell-hemolytic antibody system.

Hemolytic antigen-antibody system:

One of our immediate objectives is to learn with respect to the F-hapten-rabbit-antibody system whether or not the ratio of hemolytic activity to antibody weight is constant. From previous work it is already known that antibody against the isophile antigen of the sheep erythrocyte displays much lower hemolytic activity per milligram of antibody N than antibody to the F-hapten. The reason for this variation requires exploration; specifically we desire to learn whether this is a matter of variation in complement-fixing ability, and, if so, why. In addition, the thermodynamics of this antigen-antibody reaction require exploration.

Long Range:

1) Continuation of efforts to elucidate the mechanism of antibody-complement action on erythrocytes; 2) Extension of this work to bacterial cells; 3) Extension to mammalian cells other than erythrocytes. In connection with studies in this laboratory on the production of poliomyelitis virus in tissue culture, facilities have become available

for the large-scale preparation in tissue culture of single cell suspensions from kidney as well as other organs. We hope to use material of this type for studying the cytotoxic action of antibody and complement; 4) Extension of the work in the direction of studying the mechanism of anaphylactic and allergic reactions. Specifically, this would proceed along essentially two lines: a) study of the production of anaphylatoxin in serum as a result of antigen-antibody interaction; b) studies of the passive sensitization of cells followed by antibody-complement action (i.e., reactions of the indirect hemolysis type).

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* During current report period.